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13. ABSTRACT (Maximum 200 Words)
The HER-2 receptor tyrosine kinase (erbB-2) and the estrogen receptor (ER) participate in the establishment and progression of breast cancer. While the anti-estrogen, tamoxifen, is an effective breast cancer therapeutic, resistance is a clinical problem. Overexpression of HER-2 (ErbB-2) ahs been found to confer poor outcome and resistance to the antiestrogen, tamoxifen. Purpose. The objective is to evaluate the effects of Herstatin an inhibitor of the HER-2 receptor, on hormonal responsiveness of breast cancer cells that overexpress compared to breast cancer cells that do not overexpress HER-2. Scope. The effects of Herstatin will be tested on MCF-7 and MCF-7/HER-2 breast carcinoma cells by stable transfection with Herstatin, or by treatment with purified exogenous Herstatin. Results. We have generated and characterized signaling properties of two MCF7 clonal cell lines stably transfected with Herstatin and two MCF7 cell lines that overexpress both HER-2 and Herstatin. We found that Herstatin expression blocked signaling and growth mediated by erbB receptors and enhanced tamoxifen sensitivity, but only in HER-2 overexpressing breast cancer cells.

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INTRODUCTION

Subject: The establishment and progression of breast cancer is controlled by receptors for estrogens (ER) and peptide growth factors (1, 2, 3). Several lines of evidence suggest that estrogen responsiveness and resistance to anti-estrogens may be influenced by cross-talk between ER and erbB receptor pathways. Overexpression of HER-2 (erbB2) and signaling triggered by the erbB growth factor, Heregulin (HRG), has been found to confer resistance to the antiestrogen, tamoxifen (4-7). The involvement of erbB receptors has led to suggestions that receptor targeted inhibitors may enhance the therapeutic efficacy of tamoxifen. We recently discovered an alternative HER-2 product called Herstatin, which binds to HER-2 and the EGF receptor (EGFR) and blocks their activation (8-10). Preliminary studies indicate that Herstatin blocks both EGF and HRG signaling in estrogen responsive MCF7 cells and therefore may enhance tamoxifen sensitivity in breast cancer cells. **Purpose:** The objective of this proposal is to thoroughly evaluate the effects of Herstatin on hormonal responsiveness of ER positive breast cancer cells. **Scope:** The proposed research will evaluate the potential therapeutic efficacy of Herstatin combined with tamoxifen in the treatment of breast cancer.

BODY

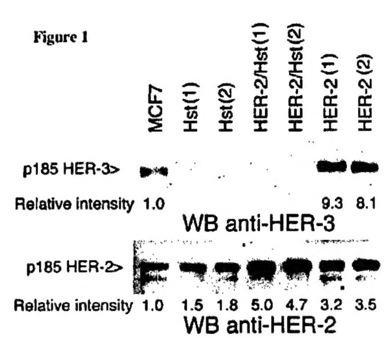
STATEMENT OF WORK

The following outlines the statement of work to be conducted and the progress we have made in this direction.

Task. Compare the tumorigenic growth of MCF7/HER-2 with MCF7/HER-2/ Hst cells.

In order to examine the impact of Herstatin on tumorigenic growth of MCF-7/HER-2 cells, we first decided it was necessary to generate and characterize two different stably transfected clonal cell lines of each. Results obtained with only one transfected cell line may be a property of the clonal selection rather than an effect of expression of Herstatin.

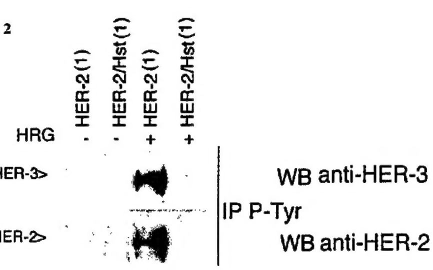
Figure 1



To accomplish this, MCF7 cells were transfected with a HER-2 expression plasmid and clonal cell lines were selected with media containing G418. Two clonal cell lines called HER-2 (1) and HER-2 (2) were selected and characterized for HER-2 expression. Figure 1 demonstrates by Western blot analysis with HER-2 antibody that these cell lines produced about three fold more HER-2 than the parental MCF7 cells. Interestingly, HER-3, the heterodimer partner of HER-2, which is important in breast cancer cell growth, was enhanced by 8-9 fold in both cell lines as shown by Western blot analysis with anti-HER-3 antibodies in Figure 1.

To develop two clonal cell lines that coexpressed HER-2 and Herstatin, the HER-2 (1) and HER-2 (2) cell lines were transfected with Herstatin and stably transfected cell lines were selected with hygromycin. Two Herstatin expressing cell lines were selected and examined for HER-3 expression (Figure 1) and for Heregulin signaling (Figure 2). Herstatin expression In the two clonal cell lines HER-2/Hst (1) and HER-2/Hst(2) resulted in complete down-regulation of HER-3 indicated by Western blot analysis (Fig. 1). This showed that HRG signaling, executed by HER-2/HER-3 heterodimers in MCF7 cells, should be blocked. In agreement, Figure 2 illustrates complete blockage of HER-2/HER-3 activation, indicated by absence of tyrosine phosphorylation in response to HRG, in one of these cells lines HER-2/Hst (1). Similar results were observed with the second clonal cell line (data not shown). These results demonstrate that both clonal cell lines that expressed Herstatin displayed a similar phenotype demonstrated by loss of HER-3 and absence of HRG signaling. Therefore prior to examining the tumorigenic growth, we established and characterized additional Herstatin expressing cell lines. Next we propose to inject nude mice with the different cell lines to examine tumor take, to determine whether MCF7/HER-2 cells exhibit tumorigenic growth in the absence of estrogen, and examine growth of xenografts with and without tamoxifen.

Figure 2



Task . Herstatin will be purified from S2 insect cells, tested for bioactivity in vitro, and stockpiled.

The S2 insect cells that are transfected with Herstatin have been used to produce Herstatin, develop a purification scheme, and characterize for bioactivity before and after freezing. The Herstatin, expressed as an N-terminally His-tagged protein in insect cells, was first purified by nickel affinity chromatography. We noticed that this protein, when analyzed on non-reducing gels contained a large component of disulfide aggregated protein that was reduced to monomeric 60 kDa Herstatin upon addition of a reducing agent. The disulfide cross-linked material was found to be inactive in bioassays. We therefore developed a gel filtration step to remove aggregated material from monomeric Herstatin. Figure 3 demonstrates the separation of monomeric Herstatin from the disulfide cross-linked and aggregated material. Figure 4 demonstrates tests of bioactivity of purified Herstatin against HRG-treated MCF7 cells. MCF7 cells were plated into 12 well plates, serum starved for 24 hrs and treated with 5nM HRG with the indicated concentrations of purified Herstatin or vehicle. The treatment was repeated at 48 hrs and the viable cells were quantitated 24 hrs later by the MTS assay. While HRG significantly stimulated growth of MCF-7 cells, Herstatin

Figure 3

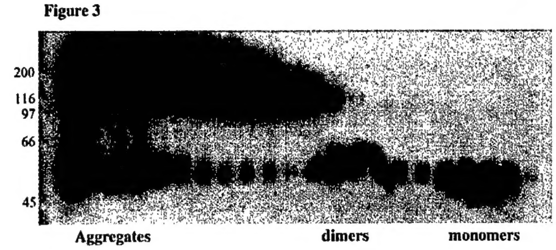
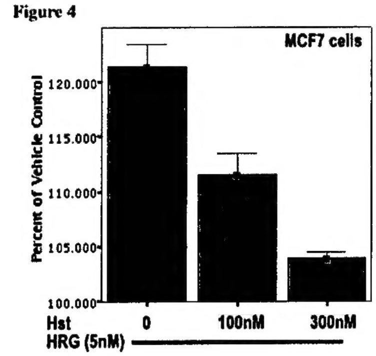


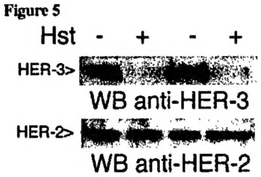
Figure 4



inhibited growth in a dose-responsive fashion. Therefore, we have developed purification and bioactivity assays and will continue to produce and stockpile purified Herstatin for studies on anti-tumor activity.

Task. Examine effects of purified Herstatin on therapeutic target, tyrosine phosphorylated HER-2 and HER-3 in MCF7-HER-2 cells.

Figure 5

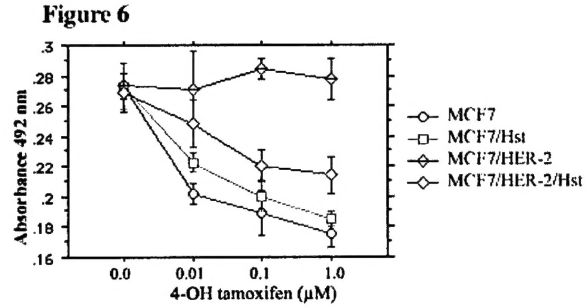


Before tests are conducted on xenografts, it is important to demonstrate that exogenous Herstatin, purified from S2 cells, effects its therapeutic target *in vitro*. Since the MCF7 cells transfected with Herstatin lost their response to HRG due to down-regulation of HER-3 receptors rather than blocked tyrosine phosphorylation (Figure 1), we investigated whether purified Herstatin may also cause loss of HER-3 receptors. The cells were treated with recombinant Herstatin and examined for levels of HER-3 by Western blot analysis (Figure 5). Exposure of the MCF7 cells to recombinant Herstatin down-regulated HER-3, but not HER-2. This further established bioactivity of recombinant Herstatin and demonstrates that down-regulation of HER-3 is an appropriate molecular target of Herstatin. In the next funding period, we will examine whether this activity of recombinant Herstatin to down-regulate HER-3 can be observed in xenograft tumor models.

Task. Examine tumorigenic growth of MCF7 and MCF-7 Herstatin with and without tamoxifen.

The purpose of this task was to approach the second major objective of this grant: To determine whether Herstatin confers tamoxifen sensitivity to breast cancer cells that DO NOT overexpress HER-2. Since Herstatin clearly blocks HRG signaling and growth as well as erbB receptor activation in MCF7 cells regardless of their HER-2 expression levels, it was possible that Herstatin may enhance sensitivity of ER positive breast cancers regardless of overexpression of HER-2. Before testing this in nude mice, it was important to first examine this *in vitro* in cultured cells. We therefore examined effects of different concentrations of 4-OH tamoxifen on growth of the different MCF7 cell lines. Figure 6 demonstrates that HER-2 overexpression in MCF7/HER-2 cells clearly conferred tamoxifen resistance relative to the parental MCF7 cells as demonstrated in numerous previous studies. Introduction of Herstatin into the HER-2 overexpressing cells (MCF7/HER-2/Hst) increased tamoxifen sensitivity, although they were less sensitive than the parental MCF7 cells (Fig. 6). The expression of Herstatin in the non HER-2 overexpressing cells (MCF7/Hst)however had no significant effect on tamoxifen sensitivity. Therefore, in the context of breast cancer cells that do not overexpress HER-2, Herstatin did not further enhance tamoxifen sensitivity even though it blocked ErbB signaling. From this study, we concluded that Herstatin may not be effective as a

Figure 6



therapeutic when combined with tamoxifen against ER positive breast cancer cells that do not overexpress HER-2.

KEY RESEARCH ACCOMPLISHMENTS

- Establish and characterize additional clonal cell lines of MCF7 cells that overexpress Herstatin and overexpress HER-2 and Herstatin.
- Determine that Herstatin expression has the same impact on ErbB receptor signaling in MCF7 cells that do or do not overexpress HER-2
- Establish that Herstatin inhibition of HRG signaling does not enhance tamoxifen sensitivity unless HER-2 is overexpressed. This answers the 2nd objective of this proposal.
- Develop protocols for Herstatin purification and bioactivity measurements.
- Determined that recombinant Herstatin, to be used in tumor models, was effective against HRG stimulated growth of MCF7 cells and that it was effective in down-modulation of the molecular target, HER-3.

REPORTABLE OUTCOMES

- Developed and characterized two clonal MCF7 cell lines that express Herstatin (MCF7/Hst) two that express HER-2 (MCF7/HER-2) and two that express bother HER-2 and Herstatin (MCF7/HER-2/Hst).

CONCLUSIONS

From studies conducted in the last funding period, we conclude that effects of Herstatin in ER positive breast carcinoma cells are reproduced in separate clonal cell lines. In addition Herstatin was found to inhibit HRG mediated growth and down-regulate the HRG receptor, HER-3, in ER positive breast cancer, regardless of HER-2 overexpression. Importantly, while Herstatin modulates ErbB receptor signaling in MCF7 cells regardless of HER-2 expression levels, Herstatin stimulates sensitivity to tamoxifen only in HER-2 overexpressing cells. These findings suggest that HER-2 targeted therapeutics may only be effective in combination with the anti-estrogen tamoxifen in ER positive breast cancer that overexpresses HER-2.

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APPENDICES

None